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Cells from Within: Intracellular Proteins

Although many applications of flow cytometry involve the staining of cells for proteins expressed on the outer membrane, cells also have many proteins that are not displayed on their surface. With appropriate procedures, flow cytometry can provide a means to analyze these intracellular proteins. The outer cell membrane is impermeable to large molecules like antibodies; however, if we intentionally fix cells to stabilize proteins and then disrupt the outer membrane, the cells can be stained with fluorochrome-conjugated monoclonal antibodies against intracellular proteins. After time to allow the antibodies to pass through the now-permeabilized membrane, the cells are washed to remove loosely bound antibodies and then are run through the flow cytometer to measure their fluorescence intensity.

This intensity should, under good conditions, be related to the amount of the intracellular protein present. However, in describing our ability to stain cells for surface proteins, we mentioned that it is best to stain viable cells. Dead cells have leaky outer membranes; they often show high nonspecific staining because antibodies get through the disrupted membrane and become trapped in the intracellular spaces. Therein lies a conflict in our ability to stain cells for intracellular proteins. Because antibodies of all types are easily trapped in the cytoplasm, there is greater potential for nonspecific staining of permeabilized cells than intact cells. The very procedure that we carry out to give access of the staining antibody to its target (intracellular) antigen actually increases the access of all antibodies to nonspecific targets. To lower this nonspecific background, antibody titers are critical and washing steps are important. Unfortunately,

even with low antibody concentrations and careful washing, background fluorescence from isotype-control antibodies is often considerably higher on permeabilized than on intact cells.

There is, in addition, a second problem. The procedures used for fixing and permeabilizing cells—to give the staining antibodies access to intracellular proteins—can modify or solubilize some antigens, thus destroying the stainability of the very proteins that are being assayed. To make matters worse, the protocol that works best for one antigen may entirely destroy a different antigen. This should not be surprising after consideration that “intracellular” includes proteins of many types and in many different environments. Some intracellular proteins are soluble, some are bound to organelle membranes, and some are in the nucleus. Therefore, methods for staining cells for intracellular proteins cannot be as standard or as dependable as the methods for staining surface proteins. They have to be individually optimized for the cells and the proteins in question.

METHODS FOR PERMEABILIZING CELLS

While not attempting to describe possible methods in detail, I feel it is important here to point out the issues involved in intracellular staining because they highlight some general issues that affect all of flow cytometric analysis. Methods for permeabilizing and fixing cells are various and must be optimized for the particular intracellular antigens being detected because some antigens are more robust than others in the face of different agents. Figure 7.1 gives an example comparing fixation/permeabilization effects on two different intracellular antigens: Five different fixation/permeabilization protocols have been used, and their effects on staining PCNA (proliferating cell nuclear antigen) and p105 (a mitosis-associated protein) have been compared. The good news is that you can stain for intracellular antigens. The bad news is that it may be difficult to stain cells optimally for two different antigens at the same time (and the relative intensity of staining for two different antigens may tell you little about the actual relative proportions of these proteins in the cell).

The general protocol for intracellular staining involves, first, staining the cells for any surface (outer membrane) antigens, as described in the previous chapter. Then the surface proteins with their

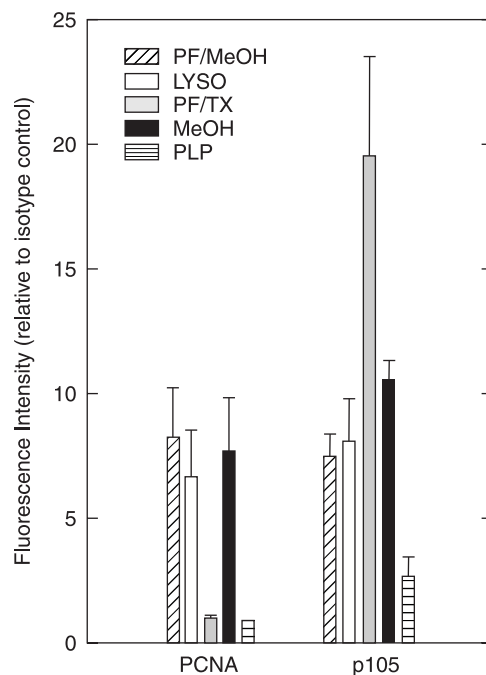


Fig. 7.1. The effects of different fixation protocols on the relative amounts detected of two different intracellular proteins. Modified from A McNally and KD Bauer as published in Bauer and Jacobberger (1994).

bound antibodies, as well as the intracellular proteins, are fixed gently to stabilize them. The purpose of the fixation is to cross-link the proteins well enough that they are not removed or washed out of the cells after the cells are permeabilized, but not so well that the intracellular antibody binding sites are masked or destroyed. Although ethanol and methanol can be used for fixation (by themselves or following another fixative), the most common fixative used prior to intracellular staining is formaldehyde. Formaldehyde is generally used at lower concentration and/or for a shorter period of time than for routine fixation of surface-stained cells (where fixation overnight in 1% formaldehyde is the [optional] last step of the procedure before flow cytometric analysis). Formaldehyde (at 0.5–1.0%) for 10 min is a good suggested concentration and time for cell fixation, but lower or higher concentrations, for shorter or longer periods of time, might be required.

This formaldehyde fixation does permeabilize the cytoplasmic membrane a bit (formaldehyde-fixed cells are permeable to small molecules), but proteins are often cross-linked too tightly for staining of intracellular proteins with antibodies. Therefore the fixation step is followed by a permeabilization step. Permeabilizing agents are usually detergents, such as Triton X-100, digitonin, NP40, or saponin, at concentrations of about 0.1%. Combined fixation/permeabilization reagents are also available as proprietary commercial reagents. With luck, the detergent will open up the cell enough so that the now-fixed proteins are accessible to the antibodies used for staining.

What are the criteria by which we can determine whether a fixation/permeabilization procedure has been optimized for an antigen in question? This optimization is, in essence, no different from optimization of a protocol for surface staining of cells. It is first necessary to maximize the fluorescence intensity of cells that are known to possess the intracellular antigen (the positive control); fixation time and concentration need to be altered in combination with different detergent concentrations to increase the positive staining. It is then necessary to decrease the background staining (using cells stained with isotype-control antibodies) as much as possible; this is done by trying increasing detergent concentrations and washing the cells thoroughly in buffer that contains the detergent. In other words, the goal is to increase the signal-to-noise ratio. Because antibody concentration, fixative agent, fixative concentration, fixation time, choice of permeabilization agent, and concentration of that permeabilizing agent are all variables in this protocol (and the optimal characteristics of each may be different for different antigens), staining for intracellular antigens requires some persistence on the part of the investigator. The following examples (in this chapter and in the following chapter on DNA) will demonstrate, however, that it is certainly possible.

EXAMPLES OF INTRACELLULAR STAINING

From the point of view of a flow cytometer, surface, cytoplasmic, and nuclear proteins are similar. The flow cytometer cannot ascertain the location of the source of fluorescence. In addition, the nuclear membrane has large enough pores that it provides little or no obstacle to staining once the outer, cytoplasmic membrane has been breached.

Cells have been stained successfully for nuclear proteins related to proliferation (for example, PCNA, Ki-67, and various cyclins, which will be discussed in the chapter on DNA) and to tumor suppression (for example, p53, c-myc, and the retinoblastoma gene product). They have also been stained for proteins bound to interior membrane surfaces (e.g., Bcl-2, multidrug resistance protein [MDR], and P-glycoprotein), and many strictly cytosolic proteins have been analyzed (like tubulin, hemoglobin, surface proteins that exist intracellularly at various stages of differentiation, and many cytokines).

As an example of one of the more complex biological situations, we can use the staining of cytokines as an illustration. Cytokines are a diverse class of proteins that, in response to cell stimulation, are synthesized and then secreted by leukocytes. For example, when T lymphocytes are stimulated, either nonspecifically or by immunological triggers, they begin to synthesize interferon- γ in their endoplasmic reticulum, send the proteins to the Golgi apparatus, and then secrete the molecules into the environment for stimulation of neighboring cells. To stain for intracellular interferon- γ , the usual technique is to stimulate cells with a biological trigger and then to incubate them with an inhibitor (brefeldin A or monensin) for several hours. These inhibitors block the normal secretion of proteins from the Golgi apparatus and thus allow the cytokine concentration to build up in the cell to levels that are detectable. After the incubation period, the cells are stained for any surface antigens of interest, fixed briefly in formaldehyde, permeabilized with saponin, and, finally, stained with a monoclonal antibody against interferon- γ .

Figure 7.2 shows an example of the way in which cells can be stained for a phenotypic surface marker (CD8) as well as the intracellular cytokine, interferon- γ . The flow data indicate that interferon- γ is associated, after PMA-ionomycin stimulation, primarily with CD8-negative cells. More of the CD8-negative than the CD8-positive cells have intracellular interferon- γ , and those that have that cytokine have more of it per cell. The tricks in the procedure for staining intracellular cytokines are as much biological as chemical (because the stain is for the end result of a functional process). In addition to a knowledge of how to fix and permeabilize a cell and how to avoid nonspecific staining, we require knowledge of how to trigger the cytokine production, knowledge of the time course of cytokine synthesis after stimulation, and knowledge of how long cells can survive

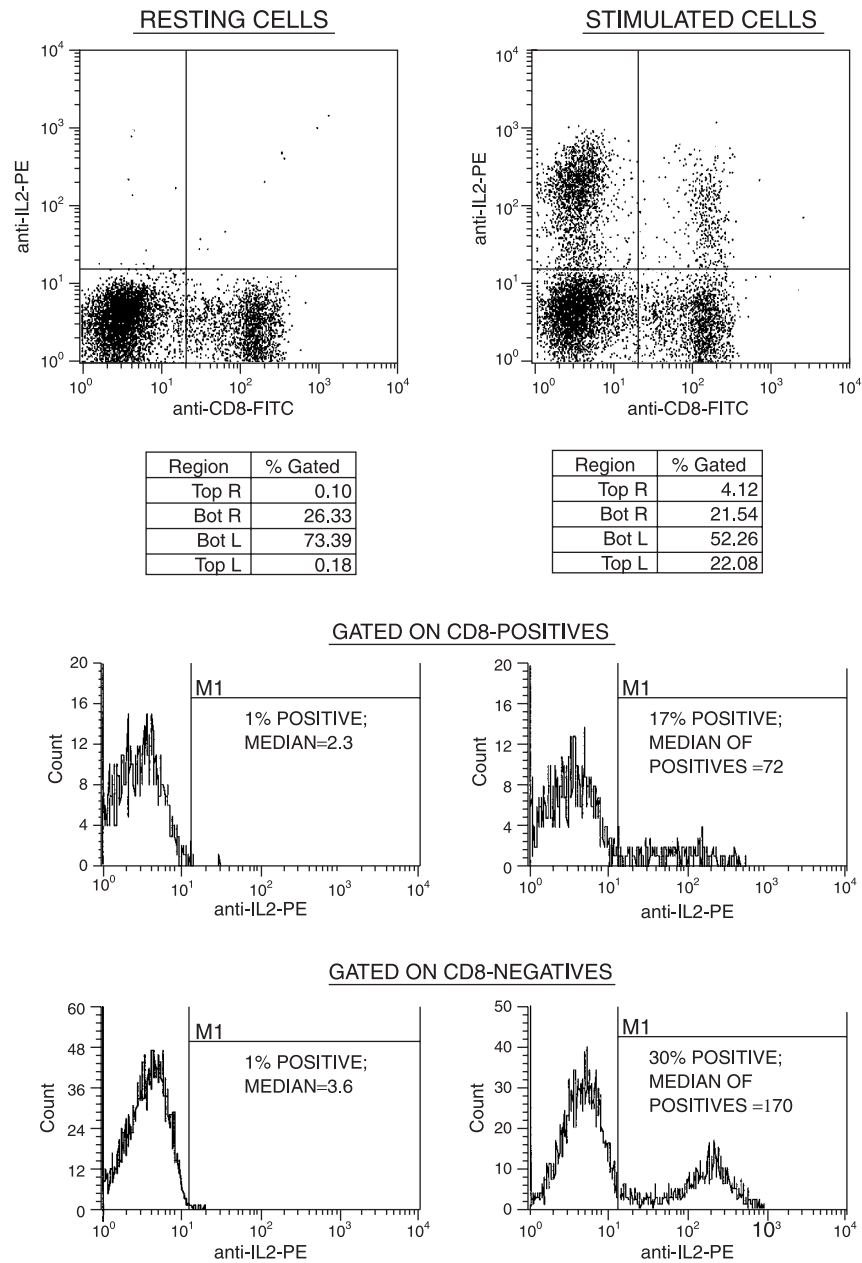


Fig. 7.2. Dot plots showing the staining of lymphocytes for intracellular interferon- γ in conjunction with an outer membrane stain (against CD8) to phenotype the cytokine-producing cells. Cells were stained for CD8 and then fixed with formaldehyde and permeabilized with saponin. The stimulus was PMA-ionomycin. Data courtesy of Paul Wallace.

with brefeldin A or monensin inhibition so that they build up large amounts of easily detectable cytokines but do not burst from this dire treatment.

As another example of intracellular staining, we can look at data from the staining of human breast tumor cells for cytokeratin and for the estrogen receptor (both intracellular proteins). Tumor cells were obtained following mastectomy by mincing and sieving the tissue to form a single-cell suspension. The suspension was then treated with saponin to permeabilize the cells. After staining for both cytokeratin and the estrogen receptor, cytokeratin-positivity selects the cells in the mixture that are of epithelial tumor origin (excluding stromal or infiltrating inflammatory cells). The two-color plot in Figure 7.3 indicates that the cytokeratin-positive (but not the cytokeratin-negative) cells express the estrogen receptor strongly (estrogen receptor positivity is associated with superior prognosis and a greater responsiveness to endocrine therapy). Gating on the cytokeratin-positive cells permits the analysis of tumor cells by themselves for the estrogen receptor without concern about the variable contamination of tumor cells by stromal cells in different samples.

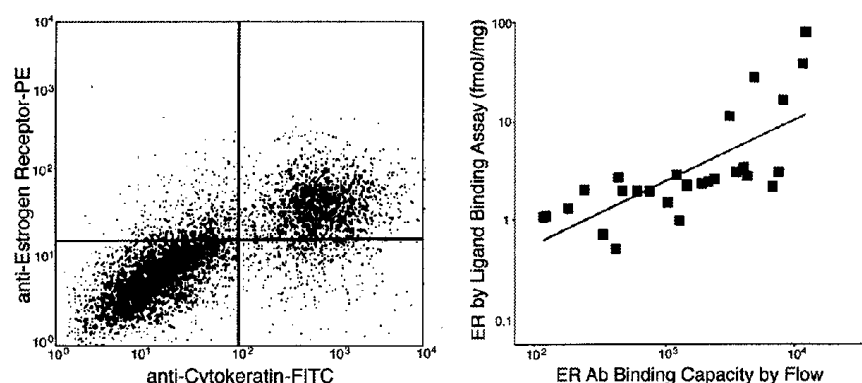


Fig. 7.3. A dot plot (on the left) showing the staining of cells from a human breast tumor for two intracellular proteins. Cytokeratin-positivity marks tumor cells in the suspension, and estrogen receptor positivity on these cells indicates superior prognosis. The plot on the right shows a correlation (in 27 breast tumors) between the intensity of estrogen receptor staining by flow cytometry and the level of estrogen receptor binding (by radioligand binding assay). Modified from Ian Brotherick et al. (1995).

Having discussed the staining of cells for both extracellular and intracellular proteins, and, in the process, learned something about general flow cytometric methodologies for analysis of data, we are now ready, in the next chapter, to apply some of these general methods to cellular components that are not proteins at all.

FURTHER READING

Chapters 12 and 13 in **Darzynkiewicz**, Chapter 15 in **Stewart and Nicholson**, and Chapter 10 in **Bauer et al.** are all good discussions of intracellular staining.